Original Article

Genotyping and distribution of putative virulence factors of *Staphylococcus aureus* isolated from dairy products in Shahrekord, Iran

Rasul Pajohesh [1], Elahe Tajbakhsh [1]*, Hassan Momtaz [1], Ebrahim Rahimi [2]

[1] Department of Microbiology, Faculty of Basic Sciences, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran. [2] Department of Food Hygiene, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.

Abstract

Introduction: *Staphylococcus aureus* (*S. aureus*) is an important foodborne pathogen worldwide and one of the most causes of food poisoning in dairy products. Therefore, this study aimed to study the genotype of the coagulase gene, distribution of putative virulence factors and antibiotic resistance genes of *S. aureus* strains isolated from raw milk and dairy products. **Material and methods**: In this cross-sectional study, 240 samples of raw milk traditional dairy products were collected in Shahrekord, Iran and was used to identify *S. aureus* by biochemical tests and PCR. Biofilm assays and Kirby–Bauer disc diffusion method were performed. Genes were detected by PCR and coagulase gene (*coa* gene) polymorphism was detected by RFLP. **Results**: Among milk and dairy product samples, 28.88% and 12.66% were positive for *S. aureus*, respectively. Amplification of the coagulase gene showed 18 strains have three bands: 320, 490, and 160 bp (genotype I) and 16 strains have two bands: 490 and 240 bp (genotype VIII). Most of the isolates were resistant to penicillin G (91.11%), ampicillin (66.66%) and oxacillin (55.55%). There was a significant relationship between biofilm formation and antibiotic resistance in *S. aureus* isolates ($p<0.05$). There was a significant relationship between the frequency of virulence genes and biofilm production in milk and dairy products ($p<0.05$). **Conclusion**: The results showed that a large percentage of milk and dairy product samples were infected with *S. aureus* and infected samples were highly resistant to antibiotics. There was a relationship between biofilm production and gene virulence and antibiotic resistance.

Keywords: *Staphylococcus aureus*, Milk, dairy products, Coagulase, biofilm, virulence

Introduction

It is believed that milk supports the growth of numerous microorganisms such as *S. aureus* because it contains many important nutrients such as proteins, vitamins, and minerals. *Staphylococcus aureus* is the most common microorganism causing staphylococcal food poisoning because it is one of the most important chief contaminant of raw milk [1-3]. In general, *Staphylococcus aureus* in raw milk originates from cows with mastitis, handlers or underprovided hygiene. It significantly contaminates the milk in favorable conditions. Its intake threatens human health, which has worried researchers because these bacteria produce toxins that lead to toxic foodborne infections [4]. Counting more than 10^3CFU in milk increases the risk of staphylococcal toxin production and resistance to pasteurization by heating [5]. It is probable that staphylococcal enterotoxins (SEs) produced by some isolates of *S. aureus* lead to food poisoning by consuming preformed SE food [6-8]. So far, 22 SEs have been explained, designated SEA to SE/V, based on their discovery chronological order [9]. *S. aureus* is associated with some enterotoxigenic toxins such as the classic serotypes SEA, SEB, SEC, SED, and SEE [10-12]. It is believed that the antimicrobial resistance of *S. aureus* postpones the antibiotic treatment of bovine mastitis [13]. Following the introduction of penicillin as an antimicrobial agent for the first time, it was shown that all strains of *S. aureus* are susceptible to it. Mutation, clonal evolution, and horizontal genes or plasmid transmission are among factors that increase antibiotic resistance and virulence features of *S. aureus* [14]. Various mechanisms, target site change of ribosome, metabolic pathway variation, efflux pumps, and enzymatic cleavage of antibiotics cause a variety of *S. aureus* strains in terms of antibiotic resistance [15-17].

Bovine mastitis is usually treated with β-lactams; however, their effectiveness has reduced because of developed β-lactamase encoded *blaZ* which reduces penicillin hydrolyze [14]. Another mechanism of β-lactam resistance is

Address for correspondence: Elahe Tajbakhsh, Department of Microbiology, Faculty of Basic Sciences, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.
Email: ee_tajbakhsh@yahoo.com

How to cite this article: Pajohesh, R., Tajbakhsh, E., Momtaz, H., Rahimi, E. Genotyping and distribution of putative virulence factors of *Staphylococcus aureus* isolated from dairy products in Shahrekord, Iran. Arch Pharma Pract 2019;10(1):63-75.

methicillin/oxacillin resistance, which is created by the presence of low-affinity penicillin-binding protein (PBP2a) encoded by the *mec*A gene [15, 16]. Aminoglycosides are powerful bactericidal agents that prevent the synthesis of protein by joining the 30S ribosomal subunit. Gentamicin and tobramycin are resistant to staphylococci that are most regularly combined with either β-lactam or a glycopeptide. In addition, antibiotic-resistant genes*: aacA-D* (aminoglycosides), *tetK, tetM* (tetracyclines), *ermA, ermB, ermC* (macrolide–lincosamide–streptogramin B), *msrA* (macrolides) and *linA* (lincosamides resistance) have been reported in last decade among isolates of *S. aureus* [18, 19]. These genes have been reported to contribute significantly in various functions such as *mecA* encodes for PBP2a, *aacA-D* for the bifunctional enzyme, *erm* (A and C) for rRNA methyltransferases, *msrA* for effluxing, *tetK*, and t*etM* for ribosome modification or extrusion [15-17]. Therefore, it is important to identify antibiotic-resistant genes in highly virulent *S. aureus* isolates as effective treatment policies and decisions can be applied to use antimicrobials.

This study aimed to isolate *S. aureus* from fresh raw milk and dairy products including creamy, yogurt, cheese, Kashk, and butter in Shahrekord, Iran.

Materials and Methods

Sampling

In this cross-sectional study, from May to November 2017, about 90 samples of raw milk (cow's milk, sheep's milk, and goat milk) and 150 samples of traditional dairy products (butter, yogurt, cheese, kashk, and creamy) were collected in Shahrekord, Iran. The samples were collected under aseptic conditions and transferred in a container containing ice cubes to the laboratory of microbiology at Islamic Azad University, Shahrekord Branch. 10 gr of kashk, yogurt, butter and cream and 10 ml of milk sample in aseptic condition are homogenized with 90 ml of peptone sterile water (Merck) and incubated for 24 hours at 37 °C. The Baird Parker technique was applied to isolate *S. aureus.* At Baird Parker Agar (BPA), the augmented specimens were streaked and the incubation plate was 37 °C for 24–48 hr. The black jet colonies enclosed by the white halo were considered probably *S. aureus.* Confirmation tests such as gram staining and several biochemical tests such as catalase testing, oxidase testing, the acid in various sugars, hemolysis on sheep blood Agar and DNase were performed. Coagulase test was used to verify *S. aureus* [20, 21].

Biofilm Formation Assays

S. aureus ATCC25923 (biofilm-forming) and *S. epidermidis* ATCC12228 (not biofilm-forming) were positive and negative control. As specified by Pereyra et al. (2016) spectrophotometry was applied in microplates using crystal violet staining to quantify the formation of biofilm [22]. For this purpose, a mixture was reached by adding 20 ml of bacterial log phase culture to 200 ml of fresh 1% glucose BHI using 96-well flat-bottom microtiter plates. BHI without bacteria was used as empty. The plates were put for incubation at 37 °C for 48 hr. Using aerobic conditions after each sampling, 300 mL of sterile phosphate-buffered saline was used to wash the wells three times; then, they were inverted for drainage. After that, 200 mL of methanol was added to each well and the plates were dried for 15 minutes. 150 mL of 0.1% crystalline violet solution was used for staining of sticky cells for 15 minutes and then sterile water was used twice to wash. 150 mL of 95% ethanol was used for 10 minutes to dissolve the purple crystal violet, and OD570 was prescribed for stained bacteria and control wells.

Susceptibility to Antibiotics

The Kirby-Bauer disc diffusion method, using Mueller–Hinton agar (Merck), was used following the guidelines of the standard clinical and laboratory Institute to perform antimicrobial susceptibility tests. As demonstrated by CLSI, the diffusion disc method in Agar Mueller–Hinton has been used to investigate the susceptibility of all antibiotics [23]. The procedure is as follows; *S. aureus* isolates were grown during the night on blood agar. Sterile saline water equivalent to the standard 0.5-McFarland was used to achieve the colonial suspension; then, 100 µl of suspension was poured on the media plate and the antibiotic disc was placed aseptically on the surface of the protected media plate. Subsequently, these plates were placed for incubation at 30 °C for methicillin and 35 °Cjmn for other antibiotics for 24 hr.

The following antibiotic disks were used; B-lactam antibiotics such as B-lactam antibiotics such as Methicillin (MET 5µg), Penicillin G (P 10µg), Ampicillin (AMP 25µg), Amoxycillin (AMS 30 µg), Oxacillin (OX 5µg), macrolides such as erythromycin (E 10µg), aminoglycoside antibiotics such as gentamycin (GEN 20µg), Kanamycin (K 20µg), Streptomycin (S 20µg), lincosamides such as Lincomycin (L 15µg), Clindamycin (CC 2µg). Glycopeptide antibiotics such as Vancomycin (V 10µg), Chloramphenicol (C 30µg), Tetracycline (TE 30µg), and Rifampicin (R 30µg) [24].

Inducible resistance to clindamycin

The D test was used based on the CLSI guidelines to examine the forced resistance to clindamycin. For this purpose, erythromycin disc was put with a 15 mm distance (edge to edge) from the clindamycin disc on a Mueller-Hinton agar plate, earlier protected with 0.5 McFarland standard bacterial suspensions. After incubation at night 37°C, flattening of the zone (D-shaped) around the clindamycin in the area between the two discs proved mandatory clindamycin resistance [12, 25].

DNA extraction and polymerase chain reaction (PCR)

DNA extraction kit following the manufacturer's instructions was used to extract genomic DNA from *S. aureus* isolates. As specified by Sambrook and Russell [26], total DNA was determined at an optical density of 260 nm. PCR was performed using specifically targeted primers to identify *S. aureus*.

Detection of virulence genes, enterotoxin genes, and PCR antibiotic resistance gene

Antibiotic resistance encoding genes such as *mecA, aca A-D, erm A, erm C, tet K* [24], SEs (*sea, seb, sec* and *sed*) [27], biofilm related genes (*bap*, *ica A.ica B, ica C, ica D*) *(6)*, and adhesion related genes (*fnbA, fnbB, clfA, clfB*) [17] were detected by PCR.

Applied primers sequence indicate in Tables 1 and 2, the annealing temperature and the PCR program. A DNA thermal cycler (Master Cycler Gradiant, Eppendrof, Germany) was used to perform the PCR. The ethidium bromide and electrophoresed were used in 1.5% agarose gel at 80 volts for 30 minutes to stain amplifiers. UV doc gel documentation systems (Uvitec, UK) were used to visualize and photograph PCR products. A comparison was run between PCR products and 100 bp DNA markers (Fermentas n, Germany).

Table 1: Oligonucleotide primers and Multiplex PCR applications used to amplification of the antibiotic resistance genes and virulence factors in S. aureus strains isolated from milk and dairy products.

Gene	Sequence	Accession number	Size (bp)	M-PCR Volume (50 µL)	PCR programme	Reference
Nuc	F: GCGATTGATGGTGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	X68417	270	5 µL PCR buffer 10X, 2 mM Mgcl2, 200 µM dNTP, 0.4 µM of each primers, 1 U Taq polymerase, 3 µL DNA template	Initial denaturation at 95˚C for 5 min, followed by 31 cycles of 45s at 95 ˚C, 60 s at 59 ˚C and 60s at 72 ˚C, and final extention at 72 ˚C for 5 min.	**[25]**
Ica A	F: GAC CTC GAA GTC AAT AGA GGT R: CCC AGT ATA ACG TTG GAT ACC	AY138959	814	5 µL PCR buffer 10X, 2.5 mM Mgcl2, 300 µM dNTP, 0.4 µM of each primers, 2 U Taq polymerase, 3 µL DNA template	Initial denaturation at 94˚C for 5 min, followed by 32 cycles of 60s at 94 ˚C, 60 s at 60 ˚C and 120s at 72 ˚C, and final extention at 72 ˚C for 5 min.	
Ica B	F: ATC GCT TAA AGC ACA CGA CGC R:TAT CGG CAT CTG GTG TGA CAG	AY382582	526			
Ica C	F: ATA AAC TTG AAT TAG TGT ATT R: ATA TAT AAA ACT CTC TTA ACA	AY138959	989			
Ica D	F: AGG CAA TAT CCA ACG GTA A R:GTC ACG ACC TTT CTT ATA TT	U43366	371			
Bap	F: CCCTATATCGAAGGTGTAGAATTG R:GCTGTTGAAGTTAATACTGTACCTG C	MF278360	971	5 µL PCR buffer 10X, 2.5 mM Mgcl2, 300 µM dNTP, 0.4 µM of each primers, 2 U Taq polymerase, 3 µL DNA template	Initial denaturation at 95˚C for 5 min, followed by 31 cycles of 60s at 95 ˚C, 90 s at 59 ˚C and 45s at 73 ˚C, and final extention at 72 ˚C for 7 min.	
Fnb B	F: ACGCTCAAGGCGACGGCAAAG R: ACCTTCTGCATGACCTTCTGCACCT	KY024702	197			
Fnb A	F: GATACAAACCCAGGTGGTGG R:TGTGCTTGACCATGCTCTTC	KU145264	191	5 µL PCR buffer 10X, 2.5 mM Mgcl2, 300 µM dNTP, 0.4 µM of each primers, 2 U Taq polymerase, 3 µL DNA template	Initial denaturation at 94 ˚C for 5 min, followed by 35 cycles of 60 s at 95 ˚C, 90 s at 57 ˚C and 45 s at 73 ˚C, and final extention at 72 ˚C for 7 min.	
Clf A	F: CCGGATCCGTAGCTGCAGATGCACC R: GCTCTAGATCACTCATCAGGTTGTTC AGG	CP031839	1000			
clfB	F: TGCAAGTGCAGATTCCGAAAAAAAC R:CCGTCGGTTGAGGTGTTTCATTTG	AP019306	194	5 µL PCR buffer 10X, 2 mM Mgcl2, 200 µM dNTP, 0.4 µM of each primers, 1 U Taq polymerase, 3 µL DNA template	Initial denaturation at 94 ˚C for 5 min, followed by 35 cycles of 60 s at 95 ˚C, 90 s at 56 ˚C and 45 s at 73 ˚C, and final extention at 72 ˚C for 7 min.	
See	F: CAAAGAAATGCTTTAAGCAATCTTAG GC R: CACCTTACCGCCAAAGCTG	M21319	482	5 µL PCR buffer 10X, 2 mM Mgcl2 200 µM dNTP, 0.4 µM of each primers, 1 U Taq polymerase, 3 µL DNA template	Initial denaturation at 94 ˚C for 5 min, followed by 35 cycles of 60 s at 94 ˚C, 60 s at 58 ˚C and 120 s at 72 ˚C, and final extention at 72 ˚C for 10 min.	**[28]**

Sec	F: CTTGTATGTATGGAGGAATAACAAA ACATG R: CATATCATACCAAAAAGTATTGCCGT	X05815	275			
Sea	F: GAAAAAAGTCTGAATTGCAGGGAAC A R: CAAATAAATCGTAATTAACCGAAGG TTC	M18970	560	5 µl PCR buffer 10X, 2.5 mM Mgcl2, 200 µM dNTP, 0.5 µm of each primers, 2 U Taq polymerase, 3 µl DNA template	Initial denaturation at 95 ˚C for 5 min, followed by 30 cycles of 30 s at 95 ˚C, 30 s at 51 ˚C and 60 s at 73 ˚C, and final extention at 72 ˚C for 6 min.	
Seb	F: CAATCACATCATATGCGAAAGCAG R: CATCTACCCAAACATTAGCACC	M11118	404			
Sed	F: GAATTAAGTAGTACCGCGCTAAATA ATATG R: GCTGTATTTTTCCTCCGAGAGT	M28521	492			
mecA F	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTGC	Y00688	532	5 µL PCR buffer 10X, 2 mM Mgcl2, 200 µM dNTP, 0.4 µM of each primers, 1 U Taq polymerase, 3 µL DNA template	Initial denaturation at 95 ˚C for 5 min, followed by 31 cycles of 45 s at 95 ˚C, 60 s at 55 ˚C and 60 s at 72 ˚C, and final extention at 72 ˚C for 5 min.	**[29]**
blaZ	F: TACAACTGTAATATCGGAGGG R: CATTACTCTTGGCGGTTTC	NG-05599	861	5 µL PCR buffer 10X, 2 mM Mgcl2, 200 µM dNTP, 0.4 µM of each primers, 1 U Taq polymerase, 3 µL DNA template	Initial denaturation at 95 ˚C for 5 min, followed by 31 cycles of 45 s at 95 ˚C, 60 s at 50 ˚C and 60 s at 72 ˚C, and final extention at 72 ˚C for 5 min.	
vanA	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	MH744356	732	5 µL PCR buffer 10X, 2 mM Mgcl2, 200 µM dNTP, 0.4 µM of each primers, 1 U Taq polymerase, 3 µL DNA template	Initial denaturation at 95 ˚C for 5 min, followed by 31 cycles of 45 s at 95 ˚C, 60 s at 55 ˚C and 60 s at 72 ˚C, and final extention at 72 ˚C for 5 min.	
Aac A-D	F: TAATCCAAGAGCAATAAGGGC R: GCCACACTATCATAACCACTA	M18086	227	5 µL PCR buffer 10X, 2.5 mM Mgcl2, 300 µM dNTP, 0.4 µM of each primers, 2 U Taq polymerase, 3 µL DNA template	Initial denaturation at 95 ˚C for 6 min, followed by 30 cycles of 60 s at 94 ˚C, 60 s at 55 ˚C and 45 s at 72 ˚C, and final extention at 72 ˚C for 7 min.	
Erm A	F: AAGCGGTAAACCCCTCTGA R: TTCGCAAATCCCTTCTCAAC	X03216	190	5 µL PCR buffer 10X, 2.5 mM Mgcl2, 300 µM dNTP, 0.4 µM of each primers, 2 U Taq polymerase, 3 µL DNA template	Initial denaturation at 95 ˚C for 6 min, followed by 30 cycles of 60 s at 94 ˚C, 60 s at 55 ˚C and 45 s at 72 ˚C, and final extention at 72 ˚C for 7 min.	
Erm C	F: AATCGTCAATTCCTGCATGT R: TAATCGTGGAATACGGGTTTG	V01278	299			
Tet K	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	S67449	360	5 µL PCR buffer 10X, 2.5 mM Mgcl2, 300 µM dNTP, 0.4 µM of each primers, 2 U Taq polymerase, 3 µL DNA template	Initial denaturation at 94 ˚C for 6 min, followed by 35 cycles of 60 s at 95 ˚C, 90 s at 55 ˚C and 45 s at 73 ˚C, and final extention at 72 ˚C for 7 min.	
Tet M	F: AGTGGAGCGATTACAGAA R: CATATGTCCTGGCGTGTCTA	AF117258	268			
Lin A	F: GGTGGCTGGGGGGTAGATGTATTAA CTGG R: GCTTCTTTTGAAATACATGGTATTTTT CGA	X61307	323	5 µL PCR buffer 10X, 2 mM Mgcl2, 200 µM dNTP, 0.4 µM of each primers, 1 U Taq DNA polymerase, 3 µL DNA template	Initial denaturation at 95 ˚C for 5 min, followed by 31 cycles of 45 s at 95 ˚C, 60 s at 57 ˚C and 60 s at 72 ˚C, and final extention at 72 ˚C for 5 min.	
Coa	F: CGA GAC CAA GAT TCA ACA AG R: AAA GAA AAC CAC TCA CAT CA	LC425037	730-1050	5 µL PCR buffer 10X, 2 mM Mgcl2, 200 µM dNTP, 0.4 µM of each primers, 1 U Taq polymerase, 3 µL DNA template	Initial denaturation at 95 ˚C for 5 min, followed by 31 cycles of 45 s at 95 ˚C, 60 s at 50 ˚C and 60 s at 72 ˚C, and final Extention at 72 ˚C for 5 min.	**[30]**

Coagulase gene typing by RFLP method

In order to investigate the polymorphism of the coagulase gene (*coa*), the PCR product was evaluated using the *Alu1* enzyme. Using this enzyme, based on the size of the band formed, even in some cases, up to 22 different patterns are observed. The PCR of the *coa* gene was performed using primers (Table 1). The digestion of PCR products was accompanied by an AluI for restriction analysis. For this purpose, 12.5 µl of PCR products were mixed with 10 U enzyme and 10×1.5 µl limited buffer; then, it was put for incubation at 37°C during the night [30]. The band size created in 1-9 genotypes are 970, 810, 810, 810, 890, 810-1050, 890 and 730, respectively.

Statistical analysis

Scheme of Pad Prism Version 6 Demo was used for statistical analysis. The chi-square, Independent T-test, ANOVA and Fischer's exact test were used to analyze the data. The value of $p < 0.05$ was considered significant.

Results

240 milk and dairy products of *S. aureus* were isolated from 45 samples (18.75%). Of the 90 milk samples, 26 samples (28.88%) were positive for *S. aureus* and of the 150 dairy product samples, *S. aureus* was reported in 19 cases (12.66%). The prevalence of *S. aureus* in cow's, sheep's, and goat's milk was reported to be 23.33%, 33.33%, and 30%, respectively. In dairy products such as Kashk, cream, cheese, yogurt, and butter, the prevalence of *S. aureus* was reported to be 20%, 13.33%, 20%, 6.55%, and 3.33%, respectively. The highest prevalence of *S. aureus* was observed in sheep's milk (33.33%) and the lowest prevalence of *S. aureus* in butter (3.33%). Statistical analysis was significantly correlated with Fisher's exact between the contamination of milk and dairy products (p-value <0/05).

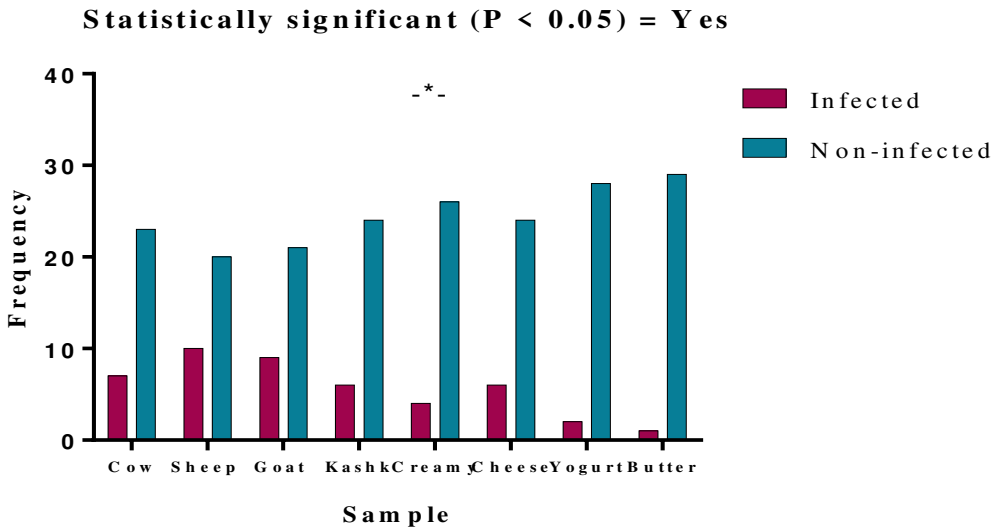

Figure 1: prevalence of *S. areus* isolated from milk and dairy products

Antibiotic resistance pattern

All 45 *S. aureus* isolates were tested for antibiotic susceptibility tests. Fourteen antimicrobial agents of different antibiotic classes were used. Most of the isolates in this study area were resistant to penicillin G (91.11%), ampicillin (66.66%), and oxacillin (55.55%). Resistance to vancomycin has not been reported in any of the isolates. The most effective antimicrobials against *S. aureus* isolates were vancomycin, which exhibited bacterial resistance (0%), gentamicin, chloramphenicol, erythromycin, rifampicin and clindamycin (4.44%), streptomycin and kanamycin (8.88%), methicillin and tetracycline (11.11%), and kanamycin (15.55%). The antibiotic resistance pattern is shown in Figure 2.

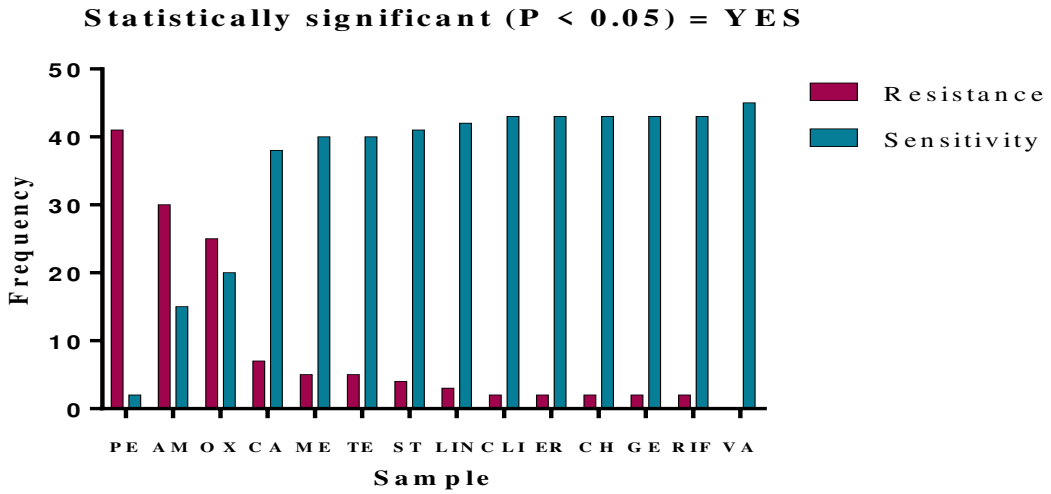

Figure 2: Antibiotic resistance pattern in *S. areus* isolates

In the microtiter plate method, 45 isolates of *S. aureus* from milk and dairy products were analyzed; 35 strains (77.77%) can form biofilms with 20 strong (44.44%) and 15 weak levels (33.33%) and 10 strains (22.22%) did not form biofilms. The prevalence of antibiotic resistance in biofilm formation and non- biofilm formation isolates are shown in table 3. Statistical analysis with Fisher's exact, there was a significant relationship between biofilm formation and antibiotic resistance (p-value <0/0001).

Table 3: Prevalence of antibiotic resistance in biofilm formation and non- biofilm formation isolates.

Antibiotic	Antimicrobial classes	Biofilm producer (N=35)		Non Biofilm producer (N=10)	
		Resistance	Sensitive	Resistance	Sensitive
Penicillin G	B-lactam	34 (97.14%)	1 (2.85%)	7 (70%)	3(30%)
Ampicillin (AM)	Amino penicillin	28 (80%)	7 (20%)	2 (20%)	8(80%)
Oxacillin	B-lactam	24 (68.57%)	9 (25.71%)	1 (10%)	9 (90%)
Methicillin	B-lactam	4 (11.42%)	31 (88.57%)	1 (10%)	9 (90%)
Vancomycin	Glycopeptide	0 (0%)	35 (100%)	0 (0%)	10 (100%)
Streptomycin	Aminoglycosides	3 (8.57%)	32 (91.42%)	1 (10%)	9 (90%)
Kanamycin	Aminoglycosides	5 (14.28%)	30 (85.71%)	2 (20%)	8 (80%)
Gentamycin	Aminoglycosides	1 (0%)	34 (97.14%)	1 (10%)	9 (90%)
Chloramphenicol	Amphenicols	2 (5.71%)	33(94.28%)	0 (0%)	10 (100%)
Lincomycin	Lincosamide	2 (5.71%)	33(94.28%)	1 (10%)	9 (90%)
Clindamycin	Lincosamide	2 (5.71%)	33(94.28%)	0 (0%)	10 (100%)
Tetracycline	Tetracycline	4 (11.42%)	31 (88.57%)	1 (10%)	9 (90%)
Erythromycin	Macrolides	2 (5.71%)	33(94.28%)	0 (0%)	10 (100%)
rifampicin	**Rifampicin**	**2 (5.71%)**	**33 (94.28%)**	**0 (0%)**	**10 (100%)**

Prevalence of antibiotic resistance genes

The most common gene for antibiotic resistance genes was *blaZ* (95.5%). The prevalence of *mecA, tetK, linA, tetM, ermA, ermB* and *Aca-D* was 22.22%, 20%, 17.77%, 15.55%, 13.33%, 11.11%, and 11.11%, respectively**.** The prevalence of antibiotic resistance in biofilm-producing isolates and non biofilm-producing isolates are shown in table 4.

Determination of virulence-associated genes

The distributions of virulence-associated genes (adhesion encoding genes, biofilm-related genes, toxin-encoding, antibiotic resistance genes) are shown in Table 3. With a range of over 70%, most of the isolates had a similar distribution of biofilm-related genes. *icaA* and *icaD* were reported in 34 isolates (75.55%), *icaC* was reported in 33 isolates (73.33%) and *icaB* was reported in 29 isolates

(64.44%). The prevalence of adhesion encoding genes, biofilm-related genes, toxin-encoding, and antibiotic resistance genes in biofilm formation and non-forming isolates are shown in Figures 3, 4 and 5.

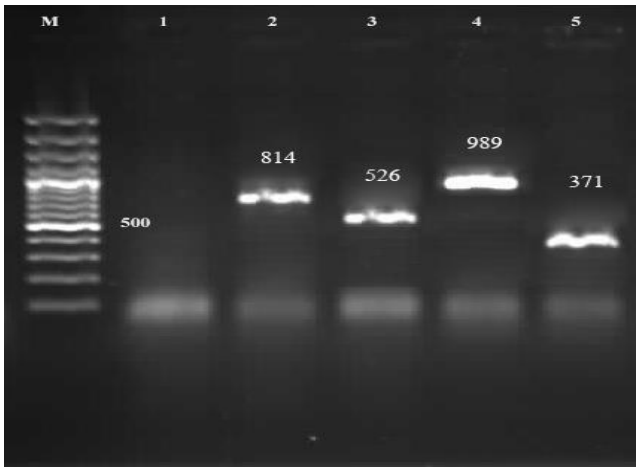

Figure 3. PCR assay results to identify *ica* genes in *S. areus* isolates. M: DNA size ladder 100 bp (Fermentas), lane 1: negative control, lane 2: *icaA*, lane 3: *icaB*, lane 4: *icaC*, lane 5: *icaD*

In adhesion encoding genes, the highest frequency was observed in *fnbA* gene (41 isolates, 91.11%). The second most abundant belongs to *fnbB* gene (36 isolates 80%). The least frequent was the *bap* gene (7 isolates, 15.55%). *clfA* and *clf B* were reported in 14 (31.11%) and 11 (24.44%) isolates.

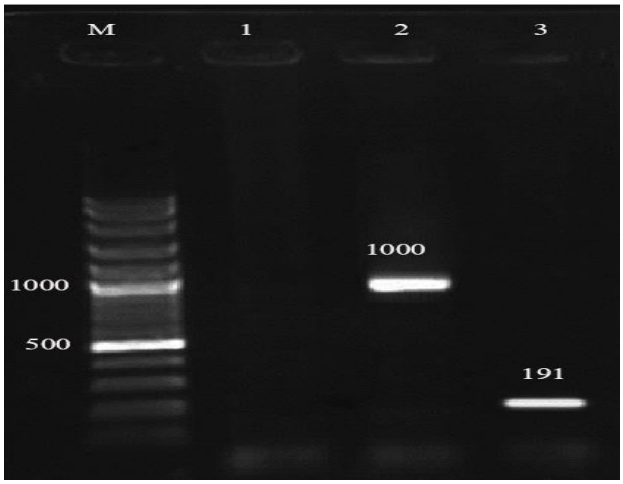

Figure 4. PCR assay results to identify *fnbA* and *clfA* genes in *S. areus* isolates. M: DNA size ladder 100 bp (Fermentas), lane 1: negative control; lane 2: *clfA*, lane 3: *fnbA*

Enterotoxin genes such as *sed, sea, sec* and *seb* were reported in 15 isolates (33.33%), 8 isolates (17.7%), 7 isolates (15.55%), and 4 isolates (8.88%), respectively. The high prevalence of *sed*, *sec,* and *seb* genes in raw milk has been reported. However, *sea* genes have been reported in dairy products.

The prevalence of adhesion encoding genes, biofilm-related genes, toxin-encoding and antibiotic resistance genes to biofilm-forming and non-forming isolates are shown in table 5.

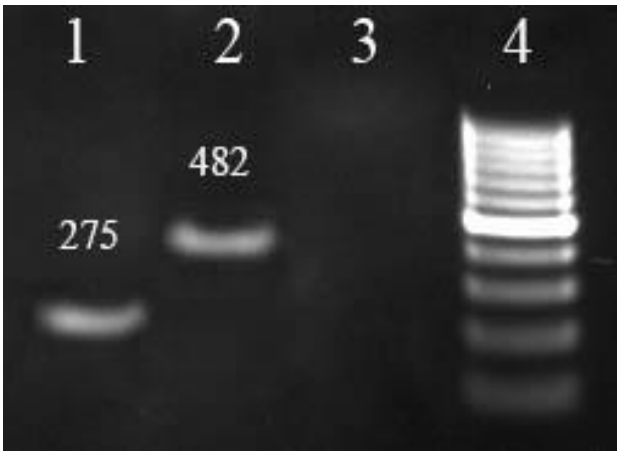

Figure 5. PCR assay results to identify *see* and *sec* genes in *S. areus* isolate. Lane 4: DNA size ladder 100 bp (Fermentas), lane 3: negative control; lane 2: *see* lane 1: *sec*

Statistical analysis with Fisher's accuracy showed a significant relationship between the frequency of genes and biofilm production in dairy (p-value = <0/0001).

Table 5: Prevalence of adhesion encoding genes, biofilm-related genes, toxin-encoding, and antibiotic resistance genes in biofilm-forming and non-forming isolates.

Functional category	No. of isolates by biofilm formation		
	Positive N= 35		Negative N=10
	Strong	Weak	
biofilm related genes	20	10	4
ica A	(100%)	(66.66%)	(40%)
ica B	20	8	1
	(100%)	(53.33%)	(10%)
ica C	20	10	3
	(100%)	(66.66%)	(30%)
ica D	20	9	5
	(100%)	(60%)	(50%)
Bap	6	1	0
	(30%)	(6.66%)	(0%)
Adhesion encoding genes	20	15	6
fnbA	(100%)	(100%)	(60%)
fnbB	20	11	5
	(100%)	(73.33%)	(50%)
clfA	10	3	1
	(50%)	(20%)	(10%)
clfB	10	1	0
	(50%)	(6.66%)	(0%)
Entrotoxin encoding genes	6	1	1
Sea	(30%)	(6.66%)	(10%)
Seb	3	1	0
	(15%)	(6.66%)	(0%)
Sec	5	1	0
	(25%)	(6.66%)	(0%)

Sed	10 (50%)	4 (26.66%)	1 (10%)
antibiotic resistance genes	20 (100%)	15 (100%)	8 (80%)
blaZ	7 (35%)	2 (13.33%)	1 (10%)
mecA	5 (25%)	2 (13.33%)	2 (20%)
tet K	5 (25%)	2 (13.33%)	1 (10%)
linA	5 (25%)	1 (6.66%)	1 (10%)
tetM	4 (20%)	1 (6.66%)	1 (10%)
erm A	3 (15%)	1 (6.66%)	1 (10%)
ermB	**3 (15%)**	**1 (6.66%)**	**0 (0%)**
Aac A-D			

Coagulase genotypes of S. *aureus* strain isolated from milk and dairy products

Following the augmentation of the coagulase gene from 45 strains isolated from *S. aureus* by specific primers, coagulase gene polymorphism was observed in 25 samples (55.55%). 18 strains (40%) had 970 bp fragment and 7 strains (15.55%) had 730 bp fragments pertinent to the *coa* gene (coagulase) in PCR. Following the enzymatic digestion with *AluI* in PCR, the products 18 strains with three bands of 490, 320, and 160 bp (genotype I) and 7 strains of two bands of 490 and 240 bp (genotype VIII). Table 6 and Figure 6 represent the results.

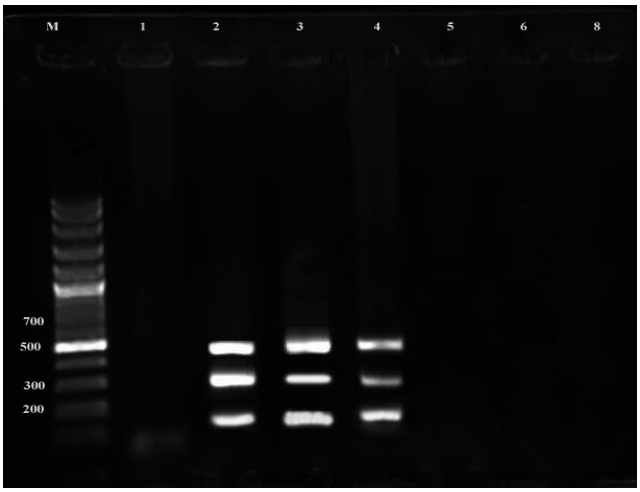

Figure 6. Agarose gel electrophoresis, RFLP from *S. aureus coa* genes isolated from raw milk and dairy product samples. Line M: 100 bp DNA ladder, line 1: negative control, lines 2–4: genotype I *coa* gene

Table 6: Coagulase genotypes of S. aureus strains isolated from dairy products and raw milk.

Genotype	PCR product (bp)	RFLP (bp)	Cow's milk	Sheep's milk	Goat's milk	Creamy	Kashk	Cheese	Yogurt	butter
I	970	490-320-160	3 (75%)	4 (66.66%)	3 (75%)	2 (100%)	2 (66.66%)	2 (66.66%)	1 (50%)	1 (100%)
II	810	410-240-160	-	-	-	-	-	-	-	-
III	810	490-240-80	-	-	-	-	-	-	-	-
IV	810	490-240-160	-	-	-	-	-	-	-	-
V	890	410-240-160-80	-	-	-	-	-	-	-	-
VI	810-1050	490-410-320-160	-	-	-	-	-	-	-	-
VII	890	490-410	-	-	-	-	-	-	-	-
VIII	730	490-240	1 (25%)	2 (33.33%)	1 (25%)	-	1 (33.33%)	1 (33.33%)	1 (50%)	-
Total			4 (100%)	6 (100%)	4 (100%)	2 (100%)	3 (100%)	3 (100%)	2 (100%)	1 (100%)

Table 7: Profiles obtained by coa gene polymorphism, virulence factors, and antimicrobial resistance pattern in S. aureus strains isolated from milk and dairy products.

Samples	Genotype	Antibiotic Resistance Phenotypes	Antibiotic Resistance Genes	Virulence Factors	
				Biofilm Producing	Non Biofilm Producing

		P10, AMP25, OX5, MET5, S20, K20	*blaZ, mecA, vanA, Aac A-D*	*icaA, icaB, icaC, icaD, bap, fnbA, fnbB, clfA*	
	I	P10, L15, OX5, AMP25	*blaZ, linA*	*icaA, icaB, icaC, icaD, fnbA, fnbB, clfA*	
	I	P10, TE30, OX, AMP	*blaZ, tetK, tetM*		
Cow's milk	I	P10, AMP25, K20	*blaZ, mecA, ermA*	*icaA, icaB, icaC, icaD, fnbA, fnbB*	
	VIII	P10, AMP25	*blaZ*	*icaA, icaB, icaC, icaD, fnbB*	
		P10, AMP25	*blaZ*	*icaA, icaB, icaD*	*icaA, icaD*
		P10, GEN20	*blaZ, Aac A-D*	*icaA, icaD*	
		P10, MET5, OX5, AMP25, CC2, TE30	*blaZ, mecA, tetK, tetM, linA*	*icaA, icaB, icaC, icaD, bap, fnbA, fnbB, clfA*	
	I	P10, L15, AMP25	*blaZ, linA*	*icaA, icaB, icaC, icaD*	
	I	P10, K20, AMP25	*blaZ, Aac A-D*	*icaA, icaB, icaC, icaD, fnbA, fnbB, clfA*	
	I	P10, AMP25, OX5	*blaZ*	*icaA, icaB icaC, icaD, fnbA, fnbB*	
Sheep's milk	I	E10	*blaZ, tetK, ermA, ermB*	*icaA, icaA, icaC, icaD, fnbA, fnbB*	
		P10, OX5	*blaZ*	*icaA, icaD*	*icaA, icaC*
	VIII	P10, OX5	*blaZ*		*icaD*
	VIII	P10, AMP25	*blaZ*		*icaB*
		P10, AMP25			
		P10, MET5, OX5, AMP25	*blaZ, mecA, tetK, tetM, linA*		
		P10, OX5, AMP25, S20	*blaZ*	*icaA, icaB, icaC, icaD, bap, fnbA, fnbB, lfA*	
	I	P10, AMP25, OX5, K20	*blaZ*	*icaA, icaB,icaC, icaD, fnbA, fnbB*	
	I	P10, AMP25, OX5	*blaZ, tetK, tetM, ermB*	*icaA,icaB, icaC, icaD, fnbB*	
Goat's milk	I	P10, OX5	*blaZ,*	*icaA, icaB, icaC, icaD*	*icaA, icaC, icaD*
		P10, AMP25	*blaZ*	*icaA, icaA, icaC, icaD*	
	VIII	P10, AMP25	*blaZ*	*icaA, icaC*	
		P10	*blaZ*		
		P10	*blaZ*		
		P10, AMP25, OX5, K20	*blaZ, mecA, tetK,*	*icaA, icaB, icaC, bap, fnbA, fnbB, clfA*	
Creamy	I	P10, AMP25, OX5	*blaZ*	*icaA, icaB, icaC, fnbB*	
	I	P10, OX5, CC2, E10	*blaZ, linA, ermA, ermB*	*icaA, icaB, icaC, icaD*	*icaC*
		P10, AMP25	*blaZ*		
	I	P10, AMP25, OX5	*blaZ, mecA, linA*	*icaA, icaB, icaC, bap, fnbA, fnbB, clfA*	
	I	P10, OX5, K20	*blaZ, ermA, ermB, Aac A-D*	*icaA, icaB, icaC, fnbB*	
Cheese		P10, OX5, TE30	*blaZ, tetK, tetM*	*icaA, icaB, icaC*	
		P10, AMP25	*blaZ*	*icaA*	
		MET5, TE30	*blaZ, mecA*		
	VIII	GEN20	*Aac A-D*		*icaD*
Yogurt	I	P10, AMP25, OX5, MET5, TE30, 20	*blaZ, mecA, tetM, Aac A-D*	*IcaA, icaB, icaC, icaD, bap, fnbA, fnbB, clfA*	
	VIII	OX5, L15	*linA evmA, ermB*	*icaC*	
Butter	I	AMP25, S20, OX5, K20	*blaZ, mecA, tetK, tetM, Aac A-D*	*icaA, icaB, icaC, fnbA, fnbB, clfA*	*icaD*

Discussion:

It is believed that S. aureus is the major cause of the zoonotic disease that can potentially transmit MRSA between livestock and humans through close contact, handling and/or consumption of *S. aureus* infected food from animals [22, 31, 32]. Contamination of dairy herds and raw milk by *S. aureus*, especially those that express the MDR phenotype and have the ability to produce biofilm and toxins [33]. The main aim of this study is to isolate and recognize *S. aureus* from raw milk and dairy products purchased from superstores and shops in Shahrekord, Iran. The prevalence of virulence factors, genotypes, and antibiotic predisposition of *S. aureus* recuperated from raw milk and dairy products such as cream, cheese, yogurt, and butter is explored. The results show that 18.75% of milk and dairy product samples were positive for *S. aureus*. Anderson et al. (2012) reported a 13.6% prevalence rate of *S. aureus*. In Germany, Schlotter et al. (2014) recognized *S. aureus* in 15.5% of all milk samples tested. However, higher recovery rates than *S. aureus* have been shown in several countries such as Zimbabwe (49.3%) [34], South Ethiopia (51.2%) [35], and Brazil (53%) [36]. The *nuc* gene is known as a genetic marker used to quickly and easily identify *S. aureus* and directly was distinguished by applying the PCR method in isolates from milk and dairy products that were first distinguished in terms of biochemical features [37, 38]. In the present study, the samples tested were found to be positive for biochemical properties for the *nuc* gene. It is believed that *S. aureus* can form biofilms, which is probably a major virulence factor determines its survival and tenacity in the environment and the host. It is believed that the formation of intercellular polysaccharide bonds through *ica* operon-encoded enzymes is frequently correlated with the formation of biofilm in *S. aureus*. Increasing resistance to various antimicrobial agents makes it difficult to treat *S.*

aureus infections; In addition, the formation of biofilm helps this organism to endure antibiotics. It has been reported that low doses of some antibiotics can force biofilm formation, indicating that exposure to biofilms may be involved in the global reaction to external stresses such as antibiotics [28, 29]. It is also believed that biofilm-producing *S. aureus* strains, especially enterotoxigenic and antibiotic-resistant ones bring about the main bacterial diseases in livestock, such as mastitis. When biofilm is formed, bacteria first join together by intercellular polysaccharide bonds and then begin to spread. The *icaADBC* operon handles the PIA and the strains having this gene cluster is believed to be the strong biofilm producers. These results are consistent with previous studies showing a higher prevalence of these genes [39].

In our study, biofilm formation was shown in 35 strains (77.77%), in 20 isolates strong biofilm reaction (44.44%) and in 15 isolates weak level (33.33%) reported. In 10 strains (22.22%) did not form biofilms. The high prevalence of biofilm-related genes was observed. For example, the prevalence of *icaA and icaD* has been reported (75.55%) and prevalence of *icaC* and *icaB* reported 73.33% and 64.44% respectively. In strong producer strains prevalence of *icaA, icaB, icaC, and icaD* have been reported 100%. In a study conducted by Vasudevan et al. (2003), a total of 35 strains of *S. aureus* isolated from bovine mastitic samples were observed in 24 samples (68.57%) of biofilm formation, but *ica* locus such as *icaA* and *icaB* were observed in all of 35 isolates (100%) [40]. These findings are in agreement with previous studies that show a higher prevalence rate of these genes. *fnb*A and *fnb*B are adhesion encoding genes and are usually associated with invasive diseases. In prevalence of *fnbA* (91.11%), *fnbB* (80%), *clfA* (31.11), and *clf B* (24.44%) were reported in adhesion encoding genes.

15.55%. the low prevalence of the *bap* gene in the present study is consistent with the results reported by Darwish and Afsour (2013) [4]. The carried out 40 isolates of *S. aureus* from bovine subclinical mastitis and reported the prevalence of the *bap* gene was 2.5%, while the *eno* gene had the highest rate. In a study by Szweda et al., (2012), 132 strains of *S. aureus* isolated from mastitis in eastern Poland had a biofilm yield of 57.6 % [41]. Both the *icaA* and *icaD* genes were found in all isolates, while no *bap* gene was found in all strains. The results of studies on the quantitative correlation between biofilm formation and antibiotic resistance were contradictory. For example, Neopane et al. (2018) argued that the biofilm-positive strains are highly resistant to multidrug and methicillin in comparison with biofilm-negative strains [42]; however, Eyoh et al. (2014) reported that the percentage of multidrug resistance among biofilm producers and non-biofilm formers for medical and nonmedical staffs did not considerably different. In the present study, the antibiotic resistance of isolates capable of producing biofilms was greater than isolates not capable of producing biofilms.

For example, resistance to penicillin, ampicillin, oxacillin, kanamycin, methicillin, and tetracycline in biofilm-producing strains reported 97.14%, 80%, 68.57%, 14.28%, 11.42%, and 11.42%, respectively. But in isolates that are unable to produce biofilms, they have lower antibiotic resistance. Resistance to methicillin reported in 5 isolates (11.11%), 4 isolates (11.42%) belongs to the biofilm producer and 1 isolate (10%) belongs to the non-biofilm producer. In Fisher's exact statistical analysis, there was a significant relationship between biofilm formation and antibiotic resistance (p-value <0/0001).

Veterinary hospitals usually use penicillin G, gentamycin, streptomycin, ampicillin, and oxacillin antibiotics. The isolated *S. aureus* population is significantly resistant to the common and these results are consistent with previous reports [15, 43]. The vancomycin-resistant isolate of *S. aureus* has not been recorded in this study, which is consistent with Kumar et al., (2010). In the present study, 11.11% isolates of *S. aureus* were resistant to methicillin, which is consistent with Hendriksen et al. (2008), Kwon et al. (2005) [44] and Lee (2003) [45]. The higher prevalence of MRSA strains has been reported in other studies [46]. According to other studies, MRSA strains are significantly prevalent. However, Kumar et al. (2010) reported a low prevalence of MRSA. Of the 45 *S. aureus* isolates, the *mecA* gene was reported to be 22.22%. *blaZ* had 95.5% prevalence. The β-lactamase enzyme is probably activated by *blaZ*, which inactivates the antibiotic by hydrolyzing the peptide link in the β-lactam ring. In this study, the *blaZ* gene was observed in all penicillin-resistant *S. aureus* isolates; However, Yang et al. (2008) reported that *blaZ* was not recorded in all of *S. aureus* isolated from bovine mastitis cases in Gansu [47].

In some isolates, point mutations lead to phenotypic resistance rather than gene acquisition. In addition, other pathways, such as biofilm formation are significantly involved in resistance mechanisms, excluding the general resistance mechanisms [48, 49]. The prevalence of *tetK, linA, tetM, ermA, ermB*, and *Aca-D* was reported to be 20%, 17.77%, 15.55%, 13.33%, 11.11%, and 11.11%, respectively. Kumar et al. [24] reported that the distribution of antibiotic-resistant genes was *linA* (51.6%), *tetK* and *tetM* (34.4%) and *aacA-D* (26.6%). Recently, the development of MDR *S. aureus*, predominantly MRSA, leading to animal and human infections, is concerning the researchers. In the present study, in 24 isolates (53.33%) MDR *S. aureus* recognized that outdoing Italy (39.4%) and Poland (23%), but significantly lower than the previous two preceding reports in Chinese (87% and 72.2%, respectively) and those in India (95%) (30,46). The genes of classical enterotoxins (*sea, seb, sec, sed, and see*) were performed in 33 (73.33%) isolates. This result is consistent with the study by Mashuf et al. (2015). In a Portuguese study, the prevalence of enterotoxigenic strains was 68.2% and another Italian study reported that 59.8% of the prevalence of *se* genes in food samples [50]. In Japan, Katsuda et al. (2005) reported 183 cases (67.8%) out of 270 enterotoxigenic *S. aureus* isolates.

Our results show that *se* genes are correlated closely with the *S. aureus* strain origin. For instance, a higher ratio of strains isolated from raw milk had *sed*, *sec,* and *sebx* genes, while strains isolated from other dairy products produced *sea* gene, that were consistent with studies in other countries [11, 51-53]. In comparison with other methods such as MLEE or PFGE, the coagulase gene RFLP method addressed here for typing of *S. aureus* isolates is much simpler. To implement this method, small amounts of crude DNA are required, and it is easy to compare this individual strains with the number of PCR-augmented gene products and the sizes of their *AluI* restriction enzyme digest fragments. As a result, we tested the isolates for in terms of *coa* presence by applying PCR. Two *coa* PCR types were achieved. The main genotype found genotype I in 16 isolates (35.55%) and genotype VIII in 6 strains (16.33%). This is the result of disagreement with other studies. Sharma et al. (2017) obtained nine *coa* PCR types [54]. There is a consensus on the accurate reason for this high rate of polymorphism among the strains. However, due to a series of 81-bp tandem repeats in the 3′end region of *coa*, the antigenic properties of the gene will likely change due to the high rate of polymorphism. As a result, it would be possible that the gene's conformity is detected as the main reason for the failure of neutral antibodies [55, 56]. In the present study, genotypes I and VIII were recorded among raw milk samples, cheese, and yogurt. However, genotype I was only recorded in cream and butter. Aslantas et al. (2007) reported that among the total genotypes, the most common genotype was the type I in Hatay (83.3%) [57]. In Gaziantep, type VIII was the most common genotype (65.4%), while types I and VIII were the most common genotypes in Hatay (29.2% and 33.3%, respectively). The coevolution of the pathogens and their host, as well as differences in reservoirs, herd managing, and environmental characteristics, can justify this distribution (2007). In general, according to our results, contamination with coagulase-positive strains of *S. aureus* was observed in raw milk and dairy product samples collected in Shahrekord. It was shown that *coa* was significantly variable and genotypes I and VIII were predominant. The RFLP pattern was significantly comparable among isolates in different regions; therefore, the isolates may be transmitted from one place to another by various means. The occurrence of enterotoxin and biofilm-producing genes in these strains endangers public health since these strains are probably found in milk-borne intoxications. In addition, multidrug-resistance and MRSA strains highlight the inappropriate use of antibiotics to handle mastitis control, which has worried health researchers around the world. However, all strains were shown to be predisposed to Vancomycin; therefore, they can be used as a drug for mastitis infections in the target areas in this study. Veterinarians may use the information gathered in this study to enhance cattle health and devise approaches to produce healthier and safer milk. Therefore, the risk of associated food poisoning is reduced and the extent of antibiotic resistance in the regions will be hindered as well. Further research is needed to investigate the relationship between the occurrence of these strains in milk and their ability to infect humans.

Conclusion:

The results showed that a large percentage of milk and dairy product samples were infected with *S. aureus* and infected samples were highly resistant to antibiotics. There was a significant relationship between biofilm production in milk and dairy products with gene virulence and antibiotic resistance.

Acknowledgments

The authors would like to thank Manochehr Momeni Shahraki for his assistance in sample collection. We also especially acknowledge the staff members of the Department of Microbiology, Faculty of Basic Sciences, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran, who have helped us with the tests. The present study has been extracted from the dissertation of Rasul Pajohesh, a Ph.D. student in the Department of Microbiology, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.

Conflicts of Interest

The author(s) indicated no conflicts of interest.

Statement of Ethics

This study was performed on samples of milk and dairy products collected from Shahrekord, so there is no ethical issue in this work.

Consent to publish

Not applicable.

Availability of data and materials

All data analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

No funding was received.

Authors' contributions

RP, ET, HM, and ER performed molecular genetic studies, participated in the primers sequence alignment and drafted the manuscript. RP and HM performed sampling and culture methods. RP and ET participated in the study design, performed the statistical analysis, writing and revising the manuscript. All authors read and approved the final version of the manuscript.

References

1. Anderson KL, Lyman R, Moury K, Ray D, Watson DW, Correa MT. Molecular epidemiology of Staphylococcus aureus mastitis in dairy heifers. J Dairy Sc. 2012; 95: 4921-4930.
2. Da Silva WP, Destro MT, Landgraf M, Franco BD. Biochemical characteristics of typical and atypical Staphylococcus aureus in

mastitic milk and environmental samples of Brazilian dairy farms. Braz J Microbiol. 2000; 31: 103-106.
3. Da Silva WP, Silva JA, MRP de Macedo, de Araújo MR, Mata MM, Gandra EA. Identification of Staphylococcus aureus, S. intermedius and S. hyicus by PCR amplification of coa and nuc genes. Braz J Microbiol. 2003; 34: 125-127.
4. Darwish SF, Asfour HA. Investigation of biofilm forming ability in Staphylococci causing bovine mastitis using phenotypic and genotypic assays. Sci World J. 2013; 1–9.
5. Gotz F, Tammy B, Karl-Heinz S. The Genera Staphylococcus and Macrococcus. Prokaryote. 2006; 4: 5–75
6. DeLeo FR, Chambers HF. Reemergence of antibiotic resistant Staphylococcus aureus in the genomics era. J Clinical Inv. 2009; 119: 2464-2474.
7. Jorgensen HJ, Mørk T, Høgasen HR, Rørvik LM. Enterotoxigenic Staphylococcus aureus in bulk milk in Norway. J App Microbiology. 2005; 99: 158-166.
8. Smith JL, Buchanan RL, Palumbo SA. Effect of food environment on staphylocaccal enterotoxin synthesis: review. J Food Prot. 1982; 46: 545-555.
9. Eyoh AB, Toukam M, Atashili J, Fokunang C, Gonsu H, Lyonga EE. Relationship between multiple drug resistance and biofilm formation in Staphylococcus aureus isolated from medical and non-medical personnel in Yaounde, Cameroon. Pan Afr Med J. 2014; 17:186.
10. Ann Principato M, Qian BF. Staphylococcal enterotoxins in the Etiopathogenesis of Mucosal Autoimmunity within the gastrointestinal tract. Toxins. 2014; 6: 1471-1489.
11. Loncarevic S, Jørgensen HJ, Løvseth A, Mathisen T, Rørvik LM. Diversity of Staphylococcus aureus enterotoxin types within single samples of raw milk and raw milk products J Appl Microbiol. 2005; 98:344-350.
12. Pereira V, Lopes C, Castro A, Silva J, Gibbs P, Teixeira P. Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of Staphylococcus aureus isolates from various foods in Portugal. Food Microbiol. 2009; 26: 278-282.
13. Oliver SP, Murinda SE. Antimicrobial resistance of mastitis pathogens. Vet Clin North. Am Food Anim Pract. 2012; 28: 165-185.
14. Olsen JE, Christensen H, Aarestrup FM. Diversity and evolution of blaZ from Staphylococcus aureus and coagulase-negative staphylococci. J Antimicrob Chemother. 2006; 57: 450-460.
15. Moon JS, Lee AR, Kang HM, Lee ES, Kim MN. Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. J Dairy Sci. 2007; 90: 1176-1185.
16. Sawant AA, Gillespie BE, Oliver SP. Antimicrobial susceptibility of coagulase-negative Staphylococcus species isolated from bovine milk. Vet Microbiol. 2009; 134: 73-81.
17. Raissy M, Moumeni M, Ansari M, Rahimi E. Antibiotic resistance pattern of some *Vibrio* strains isolated from seafood, Iranian Journal of Fisheries Sci.2012; 11: 618-626.
18. Hendriksen RS, Mevius DJ, Schroeter A, Teale C, Meunier D, Butaye P, et al. Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. Acta Vet Scand. 2008; 50:1–10
19. Lina G, Quaglia A, Reverdy ME, Leclercq R, Vandenesch F, Etienne J. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among Staphylococci. Antimicrob. Agent Chemother. 2008; 43: 1062–1066.
20. Singh P, Praksh A. Isolation of Escherichia coli, Staphylococcus aureus and Listeria monocytogenes from milk products sold under market conditions at Agra region. Acta Agr Slov. 2008; 92: 83–88
21. Solati SM, Tajbakhsh E, Khamesipour F, Gugnani HC. Prevalence of virulence genes of biofilm producing strains of Staphylococc us epidermidis isolated from clinical samples in Iran. AMB Express. 2015; 47: 1-5.
22. Pereyra EA, Picech F, Renna MS, Baravalle C, Andreotti CS, Russi R. Detection of Staphylococcus aureus adhesion and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells. Vet Microbiol. 2016; 183: 69–77.
23. CLSI. Performance standards for antimicrobial susceptibility testing; twentyfifth informational supplement. CLSI document M100-S25. Wayne: Clinical and Laboratory Standards Institute; 2017.
24. Kumar R, Yadav BR, Singh RS. Genetic determinants of antibiotic resistance in Staphylococcus aureus isolates from milk of mastitic Crossbred cattle. Curr Microbiol. 2010; 60: 379–386.
25. Kateete DP, Kabugo U, Baluku H, Nyakarahuka L, Kyobe S, Okee M. Prevalence and antimicrobial susceptibility patterns of bacteria from milkmen and cow with clinical mastitis in and around Kampala, Uganda. PLoS ONE. 2013; 8: e63413.
26. Sambrook J, Russell DW. Molecular cloning. Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 2012; 58-152.
27. Mashouf RY, Hosseini SM, Mousavi SM, Arabestani MR. Prevalence of enterotoxin genes and antibacterial susceptibility pattern of Staphylococcus aureus strains isolated from animal originated foods in West of Iran. Oman Med J. 2015; 30: 283-290.
28. Kaplan JB. Antibiotic-induced biofilm formation. Int J Artif Organs. 2011; 34: 737–751
29. Hoffman LR, Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SL. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature. 2005; 436: 1171–1175.
30. Katsuda K, Hata E, Kobayashi H, Kohmoto M, Kawashima K, Tsunemitsu H. Molecular typing of Staphylococcus aureus isolated from bovine mastitic milk on the basis of toxin genes and coagulase gene polymorphisms. Vet Microbiol. 2005; 105:301-305.
31. Salgado-Ruiz T, Rodríguez A, Gutiérrez D, Martínez B. García P, Espinoza- Ortega A, Martínez-Campos A, Lagunas-Bernabé S, Vicente F, Arriaga-Jordán C. Molecular characterization and antimicrobial susceptibility of Staphylococcus aureus from small-scale dairy systems in the highlands of Central México. Dairy Sci Technol. 2015; 95:181–196.
32. Song M, Bai Y, Xu J, Carter MQ, Shi C, Shi X. Genetic diversity and virulence potential of Staphylococcus aureus isolates from raw and processed food commodities in Shanghai. Int J Food Microbiol. 2015; 195: 1–8.
33. Wang W, Lin X, Jiang T, Peng Z, Xu J, Yi L, Li F, Fanning S, Baloch Z. Prevalence and characterization of Staphylococcus aureus cultured from raw milk taken from dairy cows with mastitis in Beijing, China. Frontiers in Microbiol. 2018; 9: 1-16.
34. Katsande S, Matope MN, Pfukenyi DM. Prevalence of mastitis in dairy cows from smallholder farms in Zimbabwe. J Vet Res. 2013; 80: 523-527.
35. Abebe R, Hatiya H, Abera M, Megersa B, Asmare K. Bovine mastitis: Prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed, South Ethiopia. BMC Vet Res. 2016; 12: 1-12
36. Guimaraes FF, Manzi MP, Joaquim SF, Richini-Pereira VB, Langoni H. Short communication: Outbreak of Methicillin-Resistant Staphylococcus aureus (MRSA)-associated mastitis in a closed dairy herd. J Dairy Sci. 2017; 100: 726-730.
37. Sahebnasagh R, Saderi H, Owlia P. The prevalence of resistance to methicillin in Staphylococcus aureus strains isolated from patients by PCR method for detection of mecA and nuc genes. Iran J Public Health. 2014; 43: 84-92.
38. Sudhaharan S, Vanjari L, Mamidi N, Ede N. Vemu. Evaluation of LAMP assay using phenotypic tests and conventional PCR for detection of nuc and mecA genes among clinical isolates of Staphylococcus Spp. J Clin Diagn Res. 2015; 9: 6-9.
39. Tang J, Chen J, Li H, Zeng P, Li L. Characterization of adhesion genes, staphylococcal nuclease, hemolysis, and biofilm formation among Staphylococcus aureus strains isolated from different sources. Foodborne Pathog Dis. 2013; 10 :757–763.
40. Vasudevan P, Nair NKM, Annamalai T, Venkitanarayanan KS. Phenotypic and genotypic characterization of bovine mastitis isolates of Staphylococcus aureus for biofilm formation. Vet Microbiol. 2003; 92: 179–185.
41. Szweda P, Schielmann M, Milewski M, Frankowska A, Jakubczak A. Biofilm Production and Presence of ica and bap genes in S. aureus strains isolated from cows with mastitis in the Eastern Poland. Polish J Microbiol. 2012; 61: 65–69.
42. Neopane P, Nepal HP, Shrestha R, Uehara O, Abiko Y. In vitro biofilm formation by Staphylococcus aureus isolated from wounds of hospital admitted patients and their association with antimicrobial resistance. Int J Gen Med. 2018; 11: 25–32.

43. Sabour PM, Gill JJ, Lepp D, Pacan JC, Ahmed R, Dingwell R, Leslie K. Molecular typing and distribution of Staphylococcus aureus isolates in Eastern Canadian dairy herds. J Clin Microbiol. 2004; 42:3449–3455.
44. Kwon NH, Park KT, Moon JS, Jung WK, Kim SH, Kim JM, Hong, SK, Koo HC, Joo YS, Park YH. Staphylococcal cassette chromosome mec (SCCmec) characterization and molecular analysis for methicillin-resistant Staphylococcus aureus and novel SCCmec subtype IVg isolated from bovine milk in Korea. J Antimicrob Chemother. 2005; 56:624–632.
45. Lee JH. Methicillin (oxacillin)-resistant Staphylococcus aureus strains isolated from major food animals and their potential transmission to humans. Appl Environ Microbiol. 2003; 69: 6489–6494
46. Tyagi A, Kapil A, Singh P. Incidence of methicillin resistant Staphylococcus aureus (MRSA) in pus samples at a tertiary care hospital, AIIMS, New Delhi. J Indian Acad Clin Med. 2008; 9:33–35.
47. Yang F, Wang Q, Wang Z, Wang L, Xiao M, Xinpu L, Luo J, Zhang S, Hongsheng L. Prevalence of blaZ gene and other virulence genes in penicillin-resistant Staphylococcus aureus isolated from bovine mastitis cases in Gansu, China. Turk J Vet Anim Sci. 2015; 39: 634-636.
48. Croes S, Deurenberg RH, Boumans MLL, Beisser PS, Neef C, Stobberingh EE. Staphylococcus aureus biofilm formation at the physiologic glucose concentration depends on the S. aureus lineage. BMC Microbiol. 2009; 9: 229.
49. Pantosti A, Sanchini A, Monaco M. Monaco. Mechanisms of antibiotic resistance in Staphylococcus aureus. Future Microbiol. 2007; 2: 323–334.
50. Normanno G, Salandra G, Dambrosio A, Quaglia NC, Corrente M, Parisi A. Occurrence, characterization and antimicrobial resistance of enterotoxigenic Staphylococcus aureus isolated from meat and dairy products consumed in Turkey. Int J Food Microbiol. 2007; 115: 290: -296.
51. Akineden Ö, Hassan AA, Schneider E, Usleber E. Enterotoxigenic properties of Staphylococcus aureus isolated from goats' milk cheese. Int J Food Microbiol. 2008; 124:211-216.
52. Morandi S, Brasca M, Lodi R, Cremonesi R, Castiglioni B. Detection of classical enterotoxins and identification of enterotoxin genes in Staphylococcus aureus from milk and dairy products. Vet Microbiol. 2007; 124:.66-72.
53. Rall VL, Vieira FP, Rall R, Vieitis RL, Fernandes AJ, Candeias JM, Cardoso KF. PCR detection of staphylococcal enterotoxin genes in Staphylococcus aureus strains isolated from raw and pasteurized milk. Vet Microbiol. 2008; 10:408-413.
54. Sharma V, Sharma S, Kumar D, Khan A, Mathur M, Sharma A. Coagulase gene polymorphism, enterotoxigenecity, biofilm production, and antibiotic resistance in Staphylococcusaureus isolated from bovine raw milk in North West India. Ann Clin Microbiol Antimicrob. 2017; 16:1-14.
55. Goh SH, Byrne S, Zhang J, Chow A. Molecular typing of Staphylococcus aureus on the basis of coagulase gene polymorphisms. J Clin Microbiol. 1992; 30:1642–1645.
56. Momtaz H, Tajbakhsh E, Rahimi E, Momeni M. Coagulase gene polymorphism of Staphylococcus aureus isolated from clinical and sub-clinical bovine mastitis in Isfahan and Chaharmahal va Bakhtiari provinces of Iran. Comp Clin Path. 2011; 20: 519–522.
57. Aslantas O, Demir C, Turutoglu H, Cantekin Z, Ergun Y, Dogruer G. Cagulase gene polymorphism of Staphylococcus aureus isolated form subclinical mastitis. Turk J Vet Anim Sci. 2007; 31:253–257.